A Thesis

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Upregulation of phospholipase D1 in the spinal cords of rats with clip compression injury

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Abstract

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This study examined phospholipase D1(PLD1) expression in the central nervous system (CNS) following clip compression spinal cord injury (SCI) in Sprague Dawley rats. After inducing SCI with a vascular clip, the

expression of PLD1 in the affected spinal cord was analyzed by western blot and immunohistochemistry. Western blot analysis showed that the expression of PLD1 gradually increased in the spinal cord at days 0.5, 1, 2, and 4 post injury. Immunohistochemistry showed that some cells, including neurons, astrocytes, and some inflammatory cells, were positive for PLD1 in the lesions at days 1 and 2 post injury. At day 4, the number of PLD1-positive cells in SCI lesions increased, largely matching the increases in ED1-positive macrophages and glial fibrillary acidic protein-positive astrocytes. At this time, macrophages expressed proliferating cell nuclear antigen in addition to PLD1. These results suggest that PLD1 expression is increased in injured spinal cords, and might be involved in the activation and proliferation of macrophages and astrocytes in SCI.

Key words: astrocytes, macrophages, PLD, spinal cord injury

CONTENTS

I. Introduction

Spinal cord injury (SCI) is postulated to include primary mechanical injury and secondary damage induced by various inflammatory responses. The neuropathological outcome of SCI is characterized by edema, axonal degeneration, the infiltration of inflammatory cells, and reactive astrogliosis, in both clip compression injury (von Euler *et al.*, 1997) and weight-drop contusion models (Basso *et al.*, 1996). Functional locomotor recovery after SCI depends on the severity of the tissue destruction. The early response after SCI is well known, and includes an increase in pro-inflammatory mediators, such as tumor necrosis factor alpha, nitric oxide, and interleukin 1 beta (Pan *et al.*, 2002). Suppression of these molecules ameliorates the severity of SCI and supports locomotor recovery after SCI (Hostettler *et al.*, 2002; Xu *et al.*, 1998). In the process of spinal cord degeneration and the subsequent regeneration, both macrophages and astrocytes play important roles by activating a variety of signals, including phospholipase D (PLD).

Recently two isoforms of PLD, PLD1 and PLD2, have been characterized at the molecular level. PLD catalyzes the hydrolysis of phosphatidylcholine (PC) to phosphatidic acid (PA) and choline (English, 1996). PLD activation through diacylglycerol may be involved in a wide range of pathophysiological processes, including inflammation, secretion, cell proliferation, mitogenesis, apoptosis, and the respiratory burst in neutrophils (Boarder *et al.*, 1994).

In neuronal tissue, several studies have shown that PLD is expressed in glial cells, such as presumed astrocytes in the rat central nervous system (Kötter *et al.*, 1999), and that PLD is upregulated in astrocytes in response to transient forebrain ischemia (Lee *et al.*, 2000). However, little is known about the expression of PLD isozymes in traumatic spinal cord injury, which is characterized by macrophage activation and astrogliosis (Basso *et al.*, 1996; von Euler *et al.*, 1997).

This study examined the pattern of PLD1 expression in the spinal cords of rats with SCI, by western blot analysis, and identified the types of cells expressing PLD 1 by immunohistochemistry.

II. Materials and Methods

1. Animals

Sprague Dawley rats were purchased from Daehan Biolink Co. (Chungbuk, Korea) and bred in our animal facility. Male rats weighing 160-200 grams, aged 7-12 weeks, were used throughout the experiments. A clip compression injury operation was performed using a modification of a previous method (von Euler *et al.*, 1997).

2. Spinal cord injury induction

The animals were anesthetized and subjected to laminectomy at T9/T10. Immediately following surgery, the spinal cord was compressed with a vascular clip (occlusion pressure, 15-20 g) (Stoelting, Wood Dale, ILL.), applied vertically to the exposed spinal cord for 1 minute. After surgery, the muscles and skin were sutured. Only a laminectomy was performed in sham-operated control rats.

The sham-operated (n=5) and SCI (n=33) rats were observed daily for locomotor function by 4 blind observers,

following the criteria of the locomotor scale of Basso *et al.* (Table 1)

Table 1. Basso, Beattie, and Bresnahan Locomotor Rating Scale (Basso *et al,* 1996)

0	No observable hindlimb(HL) movement
1	Slight movement of one or two joints, usually the hip and/or knee
2	Extensive movement of one joint or extensive movement of one joint and slight movement of one other joint
3	Extensive movement of two joints
4	Slight movement of all three joints of the HL
5	Slight movement of two joints and slight movement of the third
6	Extensive movement of two joints and slight movement of the third
7	Extensive movement of all three joints of the HL
8	Sweeping with no weight wupport or plantar place ment of the paw with no weight support
9	Plantar place ment of the paw with weight wupport in stance only (i.e., when stationary) or occasional, frequent, or consistent weight-wupported dorsal stepping and no plantar stepping
10	Occasional weight-supported plantar steps: no FL-HL coordination
11	Frequent to consistent weight-supported plantar steps and no FL-HL coordination
12	Frequent to consistent weight-supported plantar steps and occasional FL-HL coordination
13	Frequent to consistent weight-supported plantar steps and frequent FL-HL coordination
14	Consistent weight-supported plantar steps, consistent FL-HL coordination, and predominant paw position during locomotion is rotated (internally or externally) when it makes initial contact with the surface as well as just before it is lifted off at the end of stance; or frequent plantar stepping, consistent FL-HL coordination, and occasional dorsal stepping
15	Consistent plantar stepping and consistent FL-HL coordination and no toe clearance or occasional toe clearance during forward limb advancement; predominant paw position is parallel to the body at initial contact
16	Consistent plantar stepping and consistent FL-HL coordination during gait and toe clearance occurs frequently during forward limb advancement; predominant paw position is parallel at initial contact and rotated at lift off
17	Consistent plantar stepping and consistent FL-HL coordination during gait and toe clearance occurs frequently during forward limb advancement; predominant paw position is parallel at initial contact and lift off
18	Consistent plantar stepping and consistent FL-HL coordination during gait and toe clearance occurs consistently during forward limb advancement; predominant paw position is parallel at initial contact and rotated at lift off
19	Consistent plantar stepping and consistent coordinated gait, consistent toe clearance, predominant paw position is parallel at initial contact and lift off, and trunk instability; tail consistently up
20	Consistent plantar stepping and consistent coordinated gait, consistent toe clearance, predominant paw position is parallel at initial contact and lift off, and trunk instability; tail consistently up
21	Consistent plantar stepping and coordinated gait, consistent toe clearance, predominant paw position is parallel throughout stance, and consistent trunk stability; tail consistently up

3. Tissue sampling

Rats (7/group) were sacrificed for histological (n=4 each) and Western blot analyses (n=3 each) at days 0.5, 1, 2, and 4 post injury.

Experimental rats (n=3) in each group were sacrificed under ether anesthesia, and the spinal cords were removed and frozen in a deep freezer (-70 ℃) for protein analysis. Pieces of the spinal cords were processed for paraffin embedding after fixation in 4% paraformaldehyde in phosphate-buffered saline (PBS), pH 7.4.

4. Western blot analysis

Frozen spinal cords were thawed at room temperature, minced, lysed in a buffer consisting of 20 mM HEPES, pH 7.2, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 150 mM NaCl, 10 g/ml leupeptin, 10 g/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride, and then homogenized. Samples were electrophoresed under denaturing conditions by 7.5% SDS-PAGE, and then blotted onto PVDF membranes. Each blot was probed with anti-PLD1 antibody diluted in blocking solution, as reported previously (Ahn *et al*, 2001; Lee *et al*, 2000).

5. Immunohistochemistry

The PLD antisera used in this study were shown to label reactive astrocytes and macrophages in experimental autoimmune encephalomyelitis (Ahn *et al*, 2001) and astrocytes in ischemic brain injury in rats (Lee *et al*, 2000).

Five-micron sections of paraffin-embedded spinal cords were deparaffinized and allowed to react with affinity-purified anti-PLD1 antibody. To identify astrocytes and macrophages, rabbit anti-GFAP (1:800) (Dako, Copenhagen, Denmark) and ED1 (Serotec, London, U.K.) were applied, respectively. Anti-proliferating cell nuclear antigen (PCNA) antibody (Sigma) was used to demonstrate proliferative activity. The immunoreaction was visualized with an avidin-biotin peroxidase complex *Elite* kit (Vector, Burlingame, CA). Peroxidase was developed with diaminobenzidine (Vector) or aminoethyl carbazole (AEC) (Zymed, San Francisco, CA) substrate kits. Before mounting, the sections were counterstained with hematoxylin. To co-localize PLD1 and glial markers in the same section, PLD1 immunostained slides, which were bleached after photographing, were further immunoreacted

with either ED1 or anti-GFAP using the single-staining protocol described above, and the color was developed using the AEC substrate kit. The co-localization of both antigens in a single cell was readily apparent.

To visualize apoptotic cells, DNA fragmentation was detected by *in situ* terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labeling (TUNEL), as recommended by the manufacturer (Intergen, Purchase, NY).

III. Results

1. Clinical obsevation of SCI

The majority of rats with SCI showed complete paralysis in the first few days after compression, and gradually recovered locomotor function(Fig. 1). In this experiment, to examine the early response of PLD1 in the course of SCI, rats were sacrificed 12 hours, and 1, 2 and 4 days post injury. At this time, the majority of rats examined showed hindlimb paralysis (BBB scale: 0)(Basso *et al.*, 1996).

In this study, rats with complete hindlimb paralysis at the time of sacrifice were used for immunohistochemistry and Western blot analyses, as this study focused on the early response of PLD1 in compression lesion SCI.

Histologically, edema and disorganization of the white and gray matter were seen 1 day post SCI (Fig. 2, B), while no lesions were seen in the sham-operated rats (Fig. 2, A). Two days post SCI, increased extracellular space was evident, and swollen axons were seen in the areas

adjacent to the injury. Some inflammatory cells were found in the affected lesions. Four days post injury, infiltration of round cells and activated microglia was seen in the lesion, and some of the lesions contained red blood cells (Fig. 2, C). All histological fields in this study (8 fields/4 animals) showed approximately similar findings, which were largely consistent with previous reports (Basso *et al.*, 1996; von Euler *et al.*, 1997).

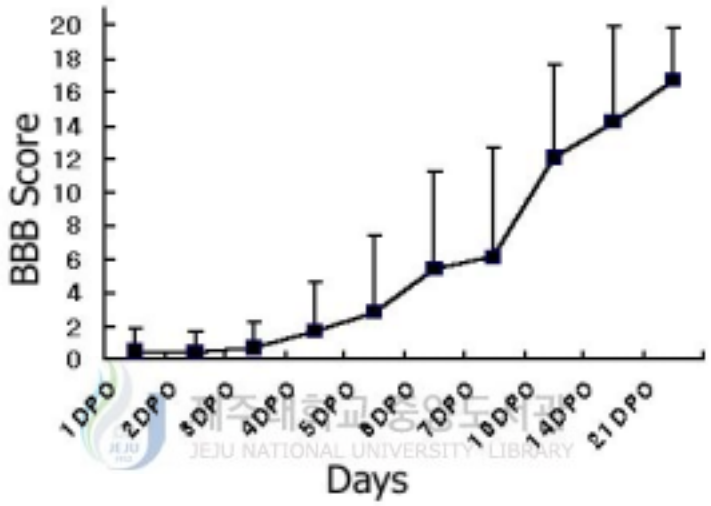

Fig. 1. The time course of the functional recovery of rats with clip compression injury operation as measured by BBB open-field locomotor score (n=33).

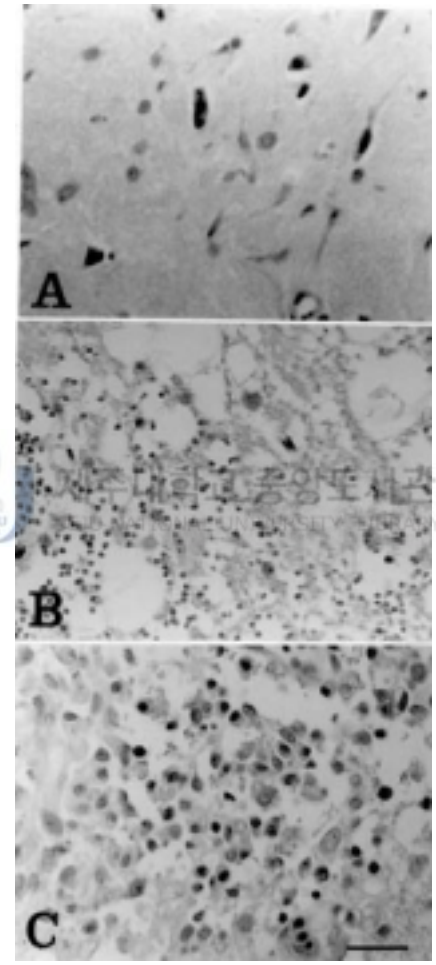

Fig. 2. Histological findings in the spinal cord of sham-operated rats (A) and SCI rats at days 1 (B) and 4 (C). The sham operations caused no direct injury to the

spinal cord. At day 1, disorganization of spinal cord tissues, including white and gray matter, was seen, while round cells were frequently found 4 days post SCI. A-C were stained with hematoxylin-eosin. Scale bar = 30 ㎛.

3. Western blot analysis

Western blot analysis showed that the level of PLD1 increased in the spinal cord at day 1 post injury, and significantly increased with time until day 4 post injury (Fig. 3). This suggests that PLD1 plays an important role in the neuropathological changes in the early stage of SCI.

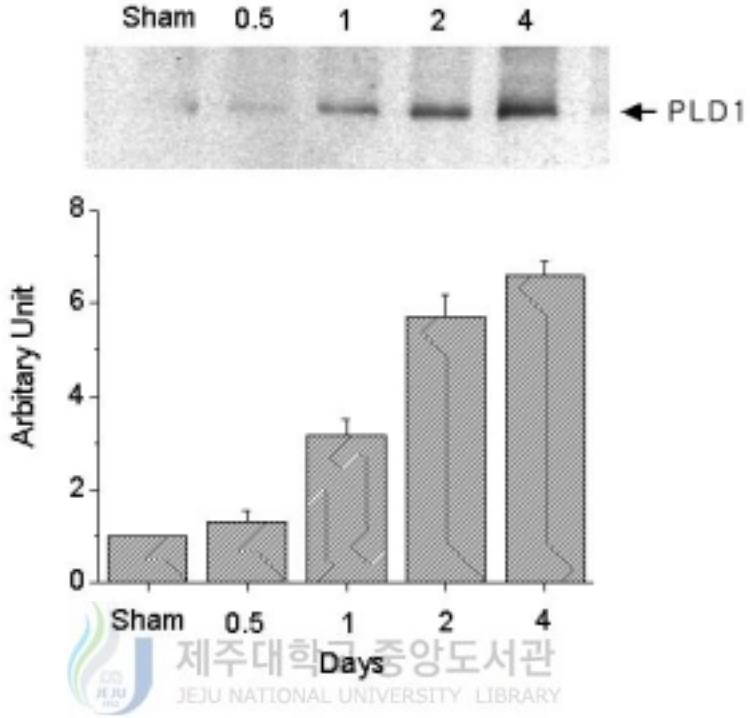

Fig. 3. Western blot analysis of PLD1 in the spinal cord of sham-operated rats (lane 1), and rats 12 hours (lane 2), and 1 (lane 3), 2 (lane 4), and 4 days (lane 5) post SCI. PLD1 was weakly expressed in the spinal cord of the sham-operated rat, while it was first expressed at 12 hours (lane 2) post SCI, and then significantly increased with time in the SCI rats. The immunoblot was quantified by densitometry, and the relative value of the control was arbitrarily defined as 1. Data points represent the mean ± S.E.M.; n=3 rats/group. Upper: a representative photo of a western blot of PLD1. The lower bar graph is based on the results of three different animals/each.

4. Immunohistochemistry

Immunohistochemistry observations confirmed the weak expression of PLD in some astrocytes and neurons in the spinal cord of sham-operated rats. At days 1 and 2 post SCI, some ED1-positive macrophages showed PLD immunoreactivity, while some astrocytes showed intense PLD1 immunostaining. At day 4 post injury, there was intense PLD (Fig. 4, C and E) staining in ED1+ macrophages (Fig. 4, F) in the lesions, while some reactive astrocytes (Fig. 4, D) showed intense PLD1 immunoreactivity. At this stage, the majority of ED1+ macrophages were PCNA-positive (Fig. 5, A), suggesting that microglia and macrophages proliferate vigorously. However, TUNEL+ cells were less common (Fig. 5, B) at this stage of SCI.

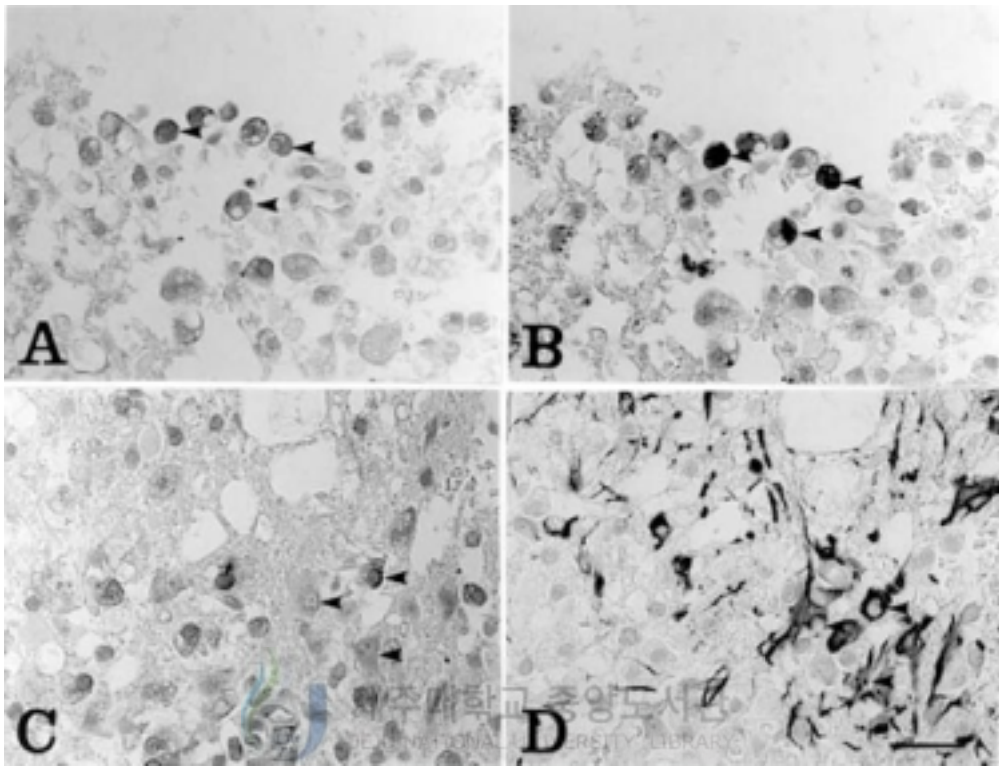

Fig. 4. Immunohistochemical study of PLD1 in the spinal cord 4 days post SCI. PLD 1(A) was found in some round cells that were positive for ED1(B). Arrowheads indicate the same cells. PLD 1 (C) was also immunostained in reactive astrocytes, which were stained with GFAP (D) in a serial section. Arrowheads in C and D indicate the same cells. A-D, counterstained with hematoxylin. A representative section from four different animals. A, DAB; B-D, AEC substrate. Scale bar = 30 ㎛.

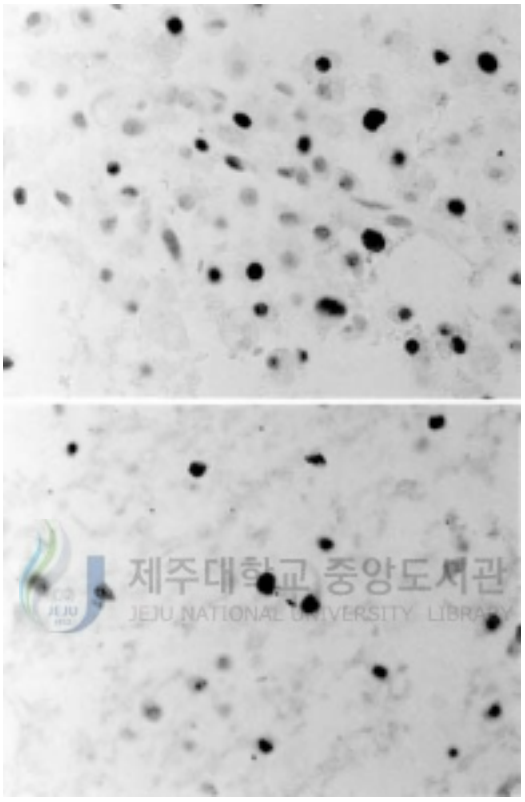

Fig. 5. Immunohistochemical staining of proliferating cell nuclear antigen and the TUNEL reaction in the spinal cord 4 days post injury. The majority of round cells at the center of the injury were positive for PCNA (A), while few TUNEL + cells (B, arrows) were seen at this time. A and B, counterstained with hematoxylin. Scale bar = 30 ㎛.

Ⅳ. Discussion

This study is the first to report that PLD1 expression is changed in the spinal cord following SCI. Recently, PLC delta4 has been identified as a gene that is down-regulated at the mRNA level after SCI using a complementary DNA microarray. This suggests that intracellular signaling mediated by PLC delta 4 is impaired or decreased in the injured spinal cord (Tachibana *et al.*, 2002). PLA2 is involved in the diffusion of glutamate after spinal cord injury (Sundstrom *et al.*, 2002); it is not known how PLA2 protein expression is regulated after SCI. In this study, we demonstrated for the first time that PLD1 is significantly up-regulated at the protein level after SCI, suggesting that PLD1 plays a role in the response to tissue damage or repair following SCI.

It is largely accepted that neuronal components, including neurons, oligodendrocytes, and microglia, undergo apoptosis after SCI (Crowe *et al.*, 1997), and this was evident in this study. Some TUNEL+ apoptotic figures were detected on cells in both the gray and white matter at 1 and 2 days post injury (data not shown). In line with a

previous paper and our results, we partly agree with the hypothesis that some host cells are eliminated through cell death either by apoptosis or necrosis. Recently, it was found that PLD is involved in the oxidative stress-induced necrosis of vascular smooth muscle cells *in vitro* (Shin et al., 2001). However, this is unlikely to match PLD1 expression and cell apoptosis at this stage (day 4 post injury) of SCI, because PLD-positive cells were more abundant in the spinal lesions in this study.

In contrast, it is postulated that PLD plays a proliferative role in microglia/ macrophages and astrogliosis at 4 days post SCI. We prefer to hypothesize that macrophages play either detrimental or beneficial roles in the course of SCI. First, macrophages function in a pro-inflammatory role because they release a variety of toxic molecules, including TNF-, nitric oxide, and other pro-inflammatory mediators in the very early stage of SCI, *i.e.*, within 24 hours. Subsequently, there are fewer of these toxic molecules 2 days post injury, suggesting that the therapeutic time window is very limited in humans.

Ironically, macrophages may play a beneficial role in tissue regeneration at 4 days post injury (Presitt *et al.*,

1997), because macrophages clean up the red blood cells and myelin debris that contain inhibitors of axonal growth, such as Nogo (GrandPre *et al.*, 2002; Wang *et al.*, 2002). We postulate that axonal sprouting starts after the myelin debris has been removed. These findings are further supported by the fact that transplantation of macrophages into spinal cord lesions can ameliorate spinal cord injury in rat models (Rapalino *et al.*, 1998). The exact role of PLD1 in macrophages in SCI needs further study.

The origin of the ED1+ cells that we observed 4 days post injury is not clear at present. We postulate that some ED1+ macrophages originate from hematogenous macrophages, localizing around vessels, as a result of hemorrhage or extravasation at the time of injury. However, it is thought that the majority of ED1+ cells in the parenchyma originate from brain macrophage/microglia via cell proliferation. This is the first SCI study to confirm the vigorous proliferation of brain macrophages with increased PLD1 expression, but it remains to be determined whether this is a cause or an effect.

The proliferative activity of PLD1 in astrocytes has been well studied in the animal model of autoimmune

encephalomyelitis (Ahn *et al.*, 2001) and in the stimulation of cultured astrocytes *in vitro* by ethanol (Kötter *et al.*, 1999). Furthermore, ethanol inhibits astroglial proliferation by inhibiting PLD (Kötter *et al.*, 1999). In SCI, astrocytes are one of the important cell types that reconstitute the damaged tissues. After the myelin debris has been removed, activated astrocytes extend their processes into the enlarged extracellular space and may release glia-derived growth factors, which enhance axonal sprouting after SCI.

Taking everything into consideration, it appears that the increased expression of PLD in lesions following SCI originates, in the main, from ED1+ macrophages and astrocytes, and that PLD plays important roles in the pathophysiology of these cell types, which are the most important cell types in the pathogenesis of the rat traumatic spinal cord disease model.

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초 록

Clip compression injury를 받은 랫트 척수 내 PLD1의 발현

(지도교수 : 신 태 균)

정 경 숙

제주대학교 대학원 수의학과

본 실험은 Sprague Dawley 랫트에서 clip compression 척수손상후의 중추신경계에서의 PLD1의 발현을 조사하였다. vascular clip으로 척수손상을 유도한 후 western blot과 면역 염색을 이용하여 손상부위의 척수내 PLD1의 발현을 분석하였다.

Western blot결과 수술후 0.5, 1, 2, 4일째의 척수에서 점차적으로 PLD1이 증가하는 것으로 나타났다. 면역염색 결과 뉴런, 별모양아교세포, 몇몇의 염증세포를 포함하는 세포들에서 손상후 1, 2일째에 양성반응을 나타내었다. 4일째에서 척수손상부위에서 PLD1 양성세포의 수는 증가하였고 glial fibrillary acidic protein 양성 별아교세포와 ED1 양성 대식세포에서 다수 일치하였다. 동시에 대식세포

는 PLD1 뿐만 아니라 proliferating cell nuclear antigen을 발현시켰다.

이상의 결과를 종합하여 볼 때, PLD1은 척수손상시 증가하고 대식세포와 별아교세포의 활성화와 증식에 관여하는 것을 알 수 있다.

주요어 : 별모양아교세포, 큰포식세포, PLD, 척수손상

감사의 글

마지막까지 부족한 저에게 학문적 가르침과 더불어 한사람의 인격체로 성장하도록 이끌어주신 신태균 교수님께 감사를 드립니다. 그리고, 저의 논문에 조언을 아끼지 않으신 이두식 교수님과 끝까지 정성껏 지도해 주신 지영흔 교수님께 감사 드립니다.

제가 수의학이란 학문을 접하기 시작하여 지금까지 저에게 많은 가르침을 주신 김희석 교수님, 박전홍 교수님, 배종희 교수님, 이경갑 교수님, 임윤규 교수님, 이영재 교수님, 정종태 교수님, 우호춘 교수님, 손원근 교수님, 이주명 교수님, 강태영 교수님, 윤영민 교수님, 주홍구 교수님, 이국경 교수님께도 감사의 마음을 전합니다.

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정 경 숙